[00132] b) For determination of the AChR content in TE671 cells grown in tissue culture, ^{125}I-α-BTX (final concentration about $2x10^{-9}$ M; 10^6 cpm) was added to a confluent cell culture in a 30 mm plate and incubated for 1 h at 37°C. The cells were then washed four times with PBS, released with 1N NaOH and cell-bound radioactivity was evaluated in a γ-counter, after deducting cpm in a control test tube containing an excess of unlabeled α-BTX (final concentration 10^{-6} M).

iv) Western blots

[00133] Electrophoresis of recombinant polypeptides corresponding to the entire or partial extracellular domain of the hAChR α-subunit and their blotting were performed essentially as described (Wilson et al., 1985; Neumann et al., 1985). The polypeptides were electrophoresed in 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was preincubated in PBS containing 0.5% hemoglobin for 1h at R.T. before addition of 10µg/ml mAbs and incubation was carried out for additional 3 h at 37°C. The membranes were washed 4 times with PBS, once with PBS containing 0.5 % Triton X-100 and then incubated for 1h at 37°C with ^{125}I-goat-anti-mouse Ig. After five washes, the blots were exposed to an X-ray sensitive film.

v) Antigenic modulation in TE671 cells

[00134] Antigenic modulation experiments were performed in 30-mm 12-well plates using TE671 cell cultures. Cells ($2x10^4$) were plated in Dulbecco Modified Eagles medium (DMEM) containing 2 mM L-glutamine, 10% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B), and grown to confluency for 72 h. The antibodies were added in triplicate to culture wells at a concentration of 1 µg/ml (and for mAbs 195 and 202 also at 5 µg/ml) for 3 h. At the end of the incubation, ^{125}I-α-BTX was added at a final concentration of $2x10^{-9}$ M (10^5 cpm) for an additional hour. AChR content was determined by measuring ^{125}I-α-BTX binding, as described in section (iii) above.

[00135] In order to test the effect of the polypeptides of the invention on the antigenic modulation induced by the antibodies, the mAbs were preincubated for 1 h at 37°C with said polypeptides (at concentrations of 10-200 µg/ml, as indicated), before their addition to the cell cultures, and the assay continued as described in section (ii) above.

vi) Passive transfer of EAMG to rats.

[00136] Lewis female rats (6 weeks old, approximate weight 120 g) were used for passive transfer experiments, as previously described (Asher et al., 1993). For the induction of

EAMG, 80 µg of the anti-MIR mAb 198 in 1 ml PBS were injected i.p. into each rat. The tested polypeptide (1 mg) was preincubated with mAb 198 for 30 min at R.T., prior to the injection into rats. The rats were observed for myasthenic symptoms and body weight. At 48 h after the administration of mAb, the animals were sacrificed and their leg muscles were removed for determination of the AChR content according to section (iii) above.

vii) Induction of EAMG and clinical evaluation

[00137] Animals were injected once in the hind foot pads with 40 µg of Torpedo AChR emulsified in complete Freund adjuvant (CFA) containing 1 mg/rat *Mycobacterium Tuberculosis* (Difco Lab., Detroit, MI). Experimental animals were weighted every week. Clinical EAMG was evaluated as follows: grade 0, no weakness or fatigability; grade 1, weak grip, fatigability; grade 2, weakness, hunched posture at rest, decrease in body weight, tremolousness; grade 3, severe weakness, marked decrease in body weight, moribund; grade 4: dead. Animals were evaluated weekly up to 7-9 weeks after immunization with Torpedo AChR. Blood samples were obtained from the retroorbital plexus.

viii) Lymphocyte proliferation assay

[00138] Popliteal lymph nodes were aseptically removed and single cell suspensions were prepared in RPMI with 10 mM HEPES. An in vitro T-lymphocyte proliferative assay in response to AChR and the different polypeptides of the invention was performed as follows: Lymph node cells were suspended in RPMI at pH 7.4 containing 10 mM HEPES, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, $5x10^{-5}$ M β-mercaptoethanol and 0.5% normal rat serum, and plated in 96-well flat bottom plates (Corning; $5x10^5$ cells/well). Increasing concentrations of antigen (0.25 to 10 µg/ml of AChR and 10 to 100 µg/ml of a recombinant polypeptide of the invention), were then added to each well. Plates were incubated at 37°C, in 7.5% CO_2 and 90% humidity. Proliferation was assayed after 3 days by measuring incorporation of thymidine-methyl-(^3H) into cells. Essentially, the cells were incubated with thymidine-methyl-(^3H) (Rotem Ind. Ltd, Beer Sheva, Israel; 0.5 mCi/2.5ml) for 24 h and then harvested and counted for radioactivity. Results are presented as incorporated cpm following subtraction of cpm in the presence of medium alone.

RESULTS

Preparation of recombinant DNA molecules

[00139] DNA molecules encoding the biologically active polypeptides Hα1-210, Hα1-121, Hα122-210, Hα1-205+p3A, Hα1-210+p3A and Hα1-121+p3A were synthesized as follows:

[00140] Total RNA was prepared as described (Asher, 1988) from the human TE671 cell line, which expresses the human muscle type nicotinic AChR (Schoepfer et al., 1988). Preparation of cDNA and the polymerase chain reaction (PCR) were performed as described (Barchan et al., 1992). The primers employed to amplify cDNA fragments corresponding to the hAChR α-subunit residue 1-210 (Hα1-210), with or without the p3A exon (Hα1-210+p3A) (Beeson et al., 1990), were constructed with sites that enabled cloning into the fusion protein expression vector pGEX-2T. The primer at the 5' end, CCGGA**TCC**GAACATGAGACC (SEQ ID NO:9), corresponds to amino acid residues 1-5 of the human AChR α-subunit sequence (nucleotides coding for the first residue are bold), and had a BamHI site (underlined). The primer at the 3' end had an EcoRI site (underlined) and was complementary to the DNA sequence coding for amino acid residues 206-210, CGGAATTCCAGGCGCTGCATGAC (SEQ ID NO:10).

[00141] In a similar way, the shorter clones Hα1-121, Hα1-121+p3A and Hα122-210 were derived by PCR using the above-

mentioned Hα1-210 and Hα1-210+p3A clones as templates. For obtaining the two DNA molecules corresponding to amino acid residues 1-121 (with and without the amino acid residues coded by the p3A exon), a primer complementary to the DNA sequence coding for amino acid residues 116-121 with an EcoRI site (underlined) CGGAATTCTGGAGGTGTCCACGTGAT (SEQ ID NO:11), was used at the 3' end. For the 5' end, the primer described above corresponding to amino acid residues 1-5 was used. For cloning of the DNA coding for Hα122-210, the primer CCGGATCC**GCC**ATCTTTAAAAGC (SEQ ID NO:12) was used at the 5' end. This primer corresponds to amino acid residues 122-126 (nucleotides coding for residue 122 are in bold) and contains a BamHI site (underlined). The primer used at the 3' end was the same as described above for the DNA molecule coding for Hα1-210 (complementary to residues 206-210). The PCR amplified DNA sequences were subcloned into the BamHI-EcoRI sites of pGEX-2T expression vector (Pharmacia) (Smith and Johnson, 1988), in frame with the GST-coding DNA sequences at the 5′ end.

[00142] The clone Hα1-205+p3A was derived by PCR, using as template the cDNA of hAChR from the TE671 cell line. The primer at the 5' end, GGCCATGGGCTCCGAACATGAGACC (SEQ ID NO:13), corresponded to amino acid residues 1-5 was designed in a way that enabled cloning into a pET8C-derived expression vector by adding a restriction site for NCO I (underlined) the initiation

codon ATG. The primer at the 3' end, CCGGATCCTCAAAAGTGRTAGGTGATRTC (SEQ ID NO:14), where R=A or G, corresponded to the complementary sequence of amino acid residues 200-205, and contained a restriction site for BamHI (underlined) and a stop codon.

[00143] All the cloned DNA molecules were sequenced in order to verify their sequence and then used to produce the recombinant polypeptides.

Preparation of recombinant polypeptides

[00144] The different recombinant DNA molecules subcloned in pGEX-2T plasmid prepared above were used to transform competent E. coli cells (strains JM101 or XL1-blue). The transformed bacteria were grown overnight in LB medium containing ampicillin, then diluted 1:150 in the medium and further grown for additional 3-5 h. Induction of fused polypeptide expression was achieved by adding 0.5 mM IPTG (isopropyl β-D-thiogalactopyranoside) for 2 h. After expression, the bacterial suspension was centrifuged, cells were lysed by freezing and thawing the pellet and resuspended in PBS (10 ml). The preparation was sonicated for five 15-sec periods, and centrifuged for 15 min at 27,000 x g. The expressed recombinant fused polypeptides were localized in the precipitate, probably in inclusion bodies. The fused

polypeptides were solubilized in 1 ml of 9 M urea, the non-soluble fraction was removed by centrifugation for 45 min at 27,000 x g, and the supernatant was diluted in 10 ml of 50 mM Tris buffer, pH 8.0 and dialyzed against the same buffer for 48 h with several changes. After ultracentrifugation for 30 min at 100,000 x g, the supernatant was divided into aliquots for storage at -80°C. The protein concentration, determined by the Lowry method, was 1-3 mg/ml, with a yield of 20-50 mg of total protein from one liter of bacterial suspension. The GST-fused polypeptides were isolated using a substrate affinity column according to Smith and Johnson, 1988. A Coomassie brilliant blue staining of the expressed GST-fused polypeptides run on 10% polyacrylamide gel is shown in Fig. 3A: from left to right, lanes 1-6, Hα1-210+p3A, Hα1-210, Hα1-121+p3A, Hα1-121, Hα122-210 and GST, appearing to have MW of 52.5, 50.0, 43.7, 41.2, 37.8 and 29.0 kD, respectively, in agreement with the expected MW calculated based on the encoded amino acid sequences of these polypeptides (see Fig.1 and Fig.2).

[00145] Expression of Hα1-205+p3A in the pET8C expression system was performed in a similar procedure using E. coli BL21 strain.

Immunochemical characterization of the recombinant polypeptides

[00146] The prepared recombinant polypeptides of were further characterized by their binding to various anti-AChR mAbs as assayed by both Western blots (Fig. 3B- mAb 198; Fig. 3C- mAb 5.5) and by ELISA (Fig. 4 and Fig. 5).

[00147] The recombinant polypeptides (20 µg each) were electrophoresed, blotted onto nitrocellulose membrane, and incubated with different mAbs as described in the Materials and Methods section (iv). Fig. 3B shows that mAb 198, which is directed to the MIR, bound to the polypeptide corresponding to the entire extracellular portion of the hAChR α-subunit (Hα1-210) and to its shorter derivative (Hα1-121), that contains the MIR, as well as to their variants including the additional p3A encoded sequence Hα1-210+p3A and Hα1-121+p3A. As expected, mAb 198 did not bind to Hα122-210, which does not include MIR, or to the GST protein itself.

[00148] The mAb 5.5, which is directed to the binding site of AChR (Mochly-Rosen and Fuchs, 1981), bound to Hα1-210, Hα1-210+p3A and to Hα122-210, all including the binding site, but it did not bind to Hα1-121, Hα1-121+p3A nor to the GST protein (Fig. 3C). As shown, both mAb 198 and mAb 5.5 bound better to the variants containing the sequence encoded by the p3A exon.

[00149] The binding of mAb 198 to the polypeptides of the invention was also determined in ELISA carried out as described in Materials and Methods section (ii), and the results are shown in Fig. 4. In this assay, as in the Western blot, mAb 198 bound better to the polypeptides Hα1-210+p3A and Hα1-121+p3A (filled symbols). Therefore, these longer variants were used in further studies. Three other anti-MIR mAbs (mAb 195, mAb 202 and mAb 35) bound to a lesser extent than mAb 198 to all tested polypeptides (not shown).

[00150] Fig. 5 illustrates the binding of various mAbs to Hα1-210+p3A: Mab 198 (filled squares) showed a very strong binding. MAb 35, which is directed against the MIR and is known to depend on the native conformation of AChR, showed very low binding to the tested polypeptides of the invention (open circles). MAb 5.5 which also depends on the native conformation of AChR, bound well to the tested polypeptides in Western blots (Fig. 3C), but to a much lesser extent than mAb 198 in ELISA (open triangles). This poor binding of mAbs 35 and 5.5 may indicate that when bound to ELISA plates only a small fraction of the recombinant polypeptide is properly folded.

[00151] Based on the results of the binding experiments in ELISA, the next step was to test whether the polypeptides of the invention bind to the mAbs also in solution. For that, the ability of the various recombinant polypeptides to inhibit the

binding of mAb 198 to Torpedo AChR was tested in ELISA. As shown in Fig 6, Hα1-210+p3A (filled squares) and Hα1-121+p3A (filled circles) inhibited this binding, with IC_{50} values of $1.8x10^{-7}M$ and $1x10^{-7}M$, respectively, whereas the GST protein (filled triangles) did not, indicating that the solubilized recombinant fused polypeptides may indeed bind to mAb 198 also in solution. As shown above (Figs. 3B and 4), the variants containing the additional 25 amino acid residues encoded by the p3A exon were more potent in inhibiting mAb 198 binding to AChR than their counterparts lacking this 25-mer.

Effect of the polypeptides on antigenic modulation of AChR in TE671 cells

[00152] Muscle AChR loss in myasthenia gravis is caused by accelerated degradation of the receptor, brought about by anti-AChR antibodies, a great portion of which are directed to the MIR. This activity of the antibodies can be demonstrated *in vitro* in cell cultures such as the human cell line TE671. This human medulloblastoma-derived cell line expresses a functional AChR which binds α-BTX and has the α-subunit of the muscle-type AChR. The ability of the recombinant polypeptides Hα1-210 and Hα1-121 to protect the AChR on TE671 cells against accelerated degradation of AChR induced by specific anti-AChR α-subunit mAbs, was examined as follows: Anti-MIR mAbs were preincubated

for 1 h at 37°C with several concentrations of the recombinant polypeptide and then added to the cells. As a control, the mAbs were preincubated with GST or with the Hα122-210 polypeptide that does not include the MIR. The inhibition effect of Hα1-121 on AChR degradation induced by mAb 198 measured as residual α-BTX binding sites, is illustrated in Fig. 7. MAb 198 causes a reduction of 41% in residual AChR following 3 h incubation with the cells (Fig. 7, lane b). Preincubation with increasing concentrations of Hα1-121 had a dose dependent protection effect against the degradation induced by mAb 198 (Fig. 7, c-g, hatched columns). At a concentration of 100 µg/ml of Hα1-121 the TE671 cells were completely protected against the accelerated AChR degradation by mAb 198. Preincubation of mAb 198 with Hα122-210, which does not contain the MIR, did not affect the antigenic modulation induced by mAb 198 and did not block AChR degradation (Fig. 7, c-g, dark columns). Hα1-210, corresponding to the entire extracellular α-subunit domain, had the same effect as the shorter fragment Hα1-121 (data not shown).

[00153] Results of a comparable experiment carried out with other anti-AChR mAbs are shown in Fig. 8. The polypeptide Hα1-121 had a similar protection effect against AChR degradation induced by two other anti-MIR mAbs, mAb 195 and mAb

202, but had a much smaller effect on mAb 35-induced AChR degradation, possibly because of the weak binding of this antibody to Hα1-121 in solution (see Fig. 5).

Modulation by the polypeptides of EAMG passively transferred by mAb 198

[00154] The effect of the polypeptides of the invention was also examined *in vivo* in a well-established animal model disease for myasthenia gravis, designated experimental autoimmune myasthenia gravis (EAMG) (Lindstrom et al., 1976 and 1976a). In animals such as rabbits, mice, guinea-pigs, monkeys and rats, EAMG can be either passively transferred by anti-AChR antibodies, or actively induced by AChR. In both cases, the treated animals show chronic symptoms of the MG disease, i.e. show general weakness, have a hunched posture, develop a flaccid paralysis of the hind limbs, have difficulties in breathing, in swallowing and in reaching food and water supplied to them, all of which result in weight loss. The animals die from respiratory insufficiency, malnutrition and dehydration. In rats, two distinct episodes of weakness occur, especially after immunization with Torpedo AChR in combination with *Mycobacterium tuberculosis* (killed) H37 Ra, with an acute phase starting 8-10 days after immunization and a chronic phase starting 3-5 weeks later.

Table 1: Recombinant fragments modulate experimental myasthenia passively transferred by a monoclonal anti-AChR antibody

Treatment	Anti-AChR mAb 198	Myasthenic symptoms	AchR content* Fmoles/mg prot.	% of control
-	-	-	39.9±6.3	100
-	+	+	19.2±3.5	48
Hα1-121	+	-	38.8±6.9	97
Hα122-210	+	+	24.5±2.4	61
GST	+	+	19.2±4.5	48
BSA	+	+	21.4±2.4	53

*Muscle AChR content was determined by α-bungarotoxin binding to AChR present in Triton X-100 extracts from rat leg muscles, 48 h after Ig administration The values (mean±SEM) are averages derived from at least three different animals.

EAMG was passively transferred in rats by mAb 198. The disease was induced within 24-48 h following administration of the antibody (Asher et al., 1993). Muscle AChR content was determined by α-bungarotoxin binding to AChR present in Triton X-100 extracts from rat leg muscles, 48 h after the mAb administration. As previously reported, the myasthenic symptoms were accompanied by a marked reduction in the muscle AChR content (48% of normal control; Table 1). In order to examine the effect of the polypeptides of the invention on the disease symptoms, mAb 198 was preincubated with a 30 fold molar excess of recombinant polypeptides of the invention, or with either GST or BSA as controls, prior to its injection into rats.

[00155] As shown in Table 1, the muscle AChR content in the EAMG-induced rats was reduced to 48% of AChR content of control untreated rats. The recombinant polypeptides of the invention were able to modulate *in vivo* muscle AChR loss and to decrease significantly clinical symptoms of EAMG. It was shown that preincubation of mAb 198 with Hα1-121+p3A prior to its injection into rats, prevented the appearance of myasthenic symptoms. The protected rats had a normal muscle AChR content (97% of control). Similar results were obtained with the Hα1-210+p3A polypeptide (data not shown). On the other hand, preincubation with either Hα122-210+p3A or with GST or BSA did

not affect the muscle AChR content significantly (61, 48 and 53% of control, respectively) and did not prevent myasthenic symptoms. Administration of Hα1-121+p3A and Hα122-210+p3A alone did not induce any myasthenic symptoms in rats.

[00156] Interestingly, similar protection effect by Hα1-121+p3A and Hα1-210+p3A was demonstrated when the recombinant polypeptide was injected together with mAb 198 without preincubation, or even two hours after the administration of mAb 198 (data not shown).

Protective effects of nasal administration of the polypeptides of the invention on actively induced EAMG in rats

[00157] Hα1-210+p3A, Hα1-121+p3A and Hα122-210 fused with GST were expressed and solubilized as described above in the preparation of recombinant polypeptides. Nasal tolerance was induced in rats by administration of a daily dose of 2.5 µg of each of said fused polypeptides in 30 µl PBS into each rat nostril, over a period of ten consecutive days. Three days later the rats were immunized with Torpedo AChR (40 µg/rat) injected into the footpads, in Complete Freund's Adjuvant supplemented with 1 mg of Mycobacterium tuberculosis $H_{37}RA$ (DIFCO). Control rats received GST instead of the recombinant polypeptide. Clinical symptoms of EAMG disease, as well as body weight, were monitored weekly. The results of the experiment

are summarized in Table 2, showing that all three tested polypeptides had a protective effect in the rats.

[00158] Rats treated intranasally with either of the three recombinant fragments, before immunization with Torpedo AChR, were protected against EAMG, as assessed by clinical symptoms of EAMG as well as by weight loss and muscle AChR content as summarized in Table 2. 67%, 56% and 34% of the rats pretreated with Hα1-210+p3A, Hα1-121+p3A and Hα122-210+p3A, respectively, were completely protected and did not develop clinical symptoms of EAMG, and the other rats in these groups were partially protected and had milder symptoms. On the other hand, all rats in the control, GST-pretreated group, were sick. As shown in Table 2, there was a marked effect of the treatment on the weight of the rats. Whereas rats in the control, GST-treated group, exhibited a notable decrease in body weight (12.8 $\pm$ 9.2 g) characteristic to EAMG, between 3 weeks and 7 weeks following AChR injection, rats in groups pretreated with AChR fragments increased significantly in their body weight. The protective effect of the nasal treatment was also evident from the receptor content data. As seen in Table 2, there was a decrease of about 55% in AChR content in the control-GST treated rats, and only 11% decrease in AChR content in rats pretreated with Hα1-210+p3A. The recombinant fragments

themselves had no myasthenogenic effects under the conditions employed for treatment. Protection against EAMG by nasal administration of the polypeptides of the invention was accompanied by a reduction in the proliferative T-cell response and IL-2 production in response to AChR (Figs. 9A-B), and in the antibody titers to both Hα1-210+p3A and to self, rat AChR (Figs. 10A-B).

Table 2: The effect of intranasal treatment with human recombinant AChR fragments on EAMG in rats.

Treatment	Clinical score[a] 0	1	2	3	4 (No/total)	Healthy rats %	Δweight 3w to 7w gr	AChR content fmoles/mg prot.	%
Control vehicle (GST)	0/10	2/10	2/10	4/10	2/10	0	-12.8±9.2	17.5±4.1	44
Hα122-210	3/9	3/9	1/9	1/9	1/9	33	+9.2±8.6	14.9±1.7	38
Hα1-121 +p3A	5/9	1/9	2/9	0/9	1/9	56	+13.6±2.5	29.6±4.5	75
Hα1-210 +p3A	6/9	1/9	1/9	0/9	1/9	67	+15.0±6.3	35.0±3.4	89
Normal rats								39.5±2.5	100

[a]Evaluated 7 weeks after the induction of EAMG.

Suppressive effects of nasal administration of the polypeptides of the invention on an ongoing EAMG

[00159] In order to evaluate the potential of the polypeptides of the invention to affect an ongoing disease, nasal administration of Hα1-210+p3A was initiated 7 days after the induction of EAMG by immunization with Torpedo AChR. At this time rats are known to be at the first, acute phase of EAMG. Other than the time of initiation, the protocol for the nasal administration was as in the previous section on protective effects.

[00160] As summarized in Table 3, suppression of EAMG was observed also when nasal treatment with Hα1-210+p3A was initiated after the induction of EAMG. Among the rats treated intranasally with Hα1-21+p3A, 30% were disease-free for at least 8 weeks following induction of EAMG, and in the other rats in the group the symptoms seemed to be milder. There was also an effect of the nasal treatment on the receptor content. As seen in Table 3, there was a loss of 68% in the AChR content in the control (treated intranasally with ovalbumin) rats, and only a 20% loss in the rats treated intranasally with Hα1-210+p3A.

Table 3: The effect of intranasal treatment with human recombinant AChR fragment Hα1-210 +p3A on ongoing EAMG in rats

Treatment[a]	Clinical score[b] 0	1	2	3	4 (No/total)	Healthy Rats %	AChR fmoles/mg protein	Content %
Control vehicle (OVA)	0/10	2/10	4/10	3/10	1/10	0	11±1	32
Hα1-210 +p3A	3/10	3/10	3/10		1/10	30	27±8.5	80
Normal rats							34±8.5	100

[a]Nasal administration was initiated 7 days after induction of EAMG by immunization with AChR and was continued for 12 consecutive days.

[b]Clinical evaluation was made 10 weeks after induction of EAMG.

Effects of oral administration of the polypeptides of the invention on EAMG in rats.

[00161] The potential of oral administration of the polypeptides of the invention to prevent EAMG was first investigated. Two recombinant preparations of the extracellular domain of human AChR α-subunit were employed for oral tolerization: Hα1-210+p3A (fused with GST), and the extracellular domain itself (Hα1-205+p3A) expressed in the pET8C expression system with no fusion protein. Rats were fed 5 times with three days interval, each time with 0.6 mg of the recombinant fragment per rat, and AChR was injected in CFA to induce EAMG, three days after the last feeding. Rats were followed clinically, as well as for weight loss for 8 weeks after EAMG induction. As shown in Fig. 11, oral feeding with either GST-fused Hα1-210+p3A or with Hα1-205+p3A had a significant protective effect on the clinical symptoms of EAMG for at least 8 weeks. The values represent the average clinical score in the group at each time point. About 70% of the rats that were pretreated orally, did not develop any clinical symptoms and the other rats in this group were partially protected. The weight of the animals corroborated with the clinical evaluation. Control, nontreated rats, lost about 10 g per rat between 4 and 8 weeks after EAMG induction, whereas rats pretreated orally with the recombinant fragments

gained about 10 g per rat during this time interval (Fig. 11). T-cell response to AChR as well as anti-rat AChR antibody titers were also reduced following oral treatment (Fig. 12).

[00162] In the second part of the experiment, the potential of oral administration of Hα1-205+p3A to modulate an ongoing disease (in rats immunized with AChR) was investigated. In this experiment, a denatured preparation of Hα1-205+p3A (designated denHα1-215+p3A) was employed for oral treatment of sick rats. Denaturation of Hα1-205+p3A was performed in 6M guanidine HCL, followed by reduction with 0.1M β-mercaptoethanol and carboxymethylation with 0.15M iodoacetamide. Rats with a mild form of EAMG (clinical score of about 1) were pooled and divided randomly into two groups. Rats in the experimental group were fed 7 times with three days interval, each time with 0.3 mg of denHα1-205+p3A per rat, and rats in the control group were fed with ovalbumin. The rats were evaluated weekly for clinical symptoms and for their body weight. As seen in Fig. 13, the disease was arrested in the rats treated orally with the recombinant fragment and their body weight increased. On the other hand the disease progressed in rats of the control group and the rats lost weight gradually.

[00163] These protection and suppression effects on EAMG shown in this Example indicate that the polypeptides of the

invention affect the autoimmune response to AChR in a manner that may be employed for immunotherapy of myasthenia gravis. Thus, the nasal or oral route of administration could provide a convenient therapeutic modality in humans.

EXAMPLE 2

[00164] The present inventors demonstrated in Example 1 that oral or nasal administration of recombinant fragments of the acetylcholine receptor (AChR) prevents the induction of experimental autoimmune myasthenia gravis (EAMG) and suppresses ongoing EAMG in rats. The present inventors have now studied in the experiments described in this example the role of spatial conformation of these recombinant fragments in determining their tolerogenicity. Two fragments corresponding to the extracellular domain of the human AChR α-subunit and differing in conformation were tested: Hα1-205 expressed with no fusion partner and Hα1-210 fused to thioredoxin (Trx-Hα1-210). The conformational similarity of the fragments to intact AChR was assessed by their reactivity with α-bungarotoxin and with anti-AChR mAbs, specific for conformation-dependent epitopes. Oral administration of the *"more native"* fragment, Trx-H1α-210, at the acute phase of disease led to exacerbation of EAMG, accompanied by an elevation of AChR-specific humoral and cellular reactivity, increased levels of Th1-type cytokines

(IL-2, IL-12), decreased levels of Th2 (IL-10) or Th3 (TGF-β) type cytokines and higher expression of costimulatory factors (CD28, CTLA4, B7-1, B7-2, CD40L and CD40). On the other hand, oral administration of the *"less native"* fragments Hα1-205 or denatured Trx-Hα1-210, suppressed ongoing EAMG and led to opposite changes in the immunological parameters. These results demonstrate that the native conformation of AChR-derived fragments renders them immunogenic and immunopathogenic and therefore not suitable for treatment of myasthenia gravis. The conformation of tolerogens should therefore be given careful attention when considering oral tolerance for treatment of autoimmune diseases. The experiments and results are presented and discussed below.

MATERIALS AND METHODS

Animals

[00165] Female Lewis rats (6-7 weeks of age) were purchased from the animal breeding center of the Weizmann Institute of Science (Rehovot, Israel).

Antigen preparation

[00166] AChR was purified from *Torpedo californica* electric organ by affinity chromatography as previously described (Aharonov et al., 1977). Recombinant fragments were synthesized by PCR on cDNA prepared from total RNA of the human

TE671 cell line. The recombinant fragment Hα1-210 containing the P3A exon (Barchan et al., 1998), was expressed as a fusion protein with thioredoxin (Trx-Hα1-210) in pThioHis-A (Invitrogen, USA) or with glutathion S-transferase (GST-Hα1-210) (Barchan et al., 1998) and Hα1-205 was expressed in pET8-C with no fusion partner. All the recombinant proteins, present in inclusion bodies, were solubilized by 9M urea followed by serial dialyses in 50mM Tris buffer, pH 8.0. Chemical modification, by reduction and carboxymethylation of recombinant fragments, was performed by reduction with 0.1M of 2-ME in 6M guanidine HCl/0.2M Tris buffer, pH 8.8, followed by blocking of the sulfhydryl groups with iodoactamide as previously described (Bartfeld et al., 1978). The denatured forms of Trx-Hα1-210 or Hα1-205 were designated denTrx-Hα1-210 and denHα1-205, respectively.

Western blot

[00167] Electrophoresis and blotting of recombinant proteins and Torpedo AChR were performed essentially as described (Barchan et al., 1998). The proteins were resolved in 12% polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking with 0.5% hemoglobin in PBS, mAb 198 (10µg/ml) was added and incubated for 2h at 37°C. The membrane was washed and then incubated for 1h at 37°C with ^{125}I-goat-

anti-mouse IgG. After washing, the blots were exposed to an X-ray-sensitive film. Binding to α-bungarotoxin (α-BTX) was detected by overlay with ^{125}I-α-BTX ($2X10^{-9}$M) followed by washing and autoradiography.

Inhibition of mAb 198 binding to AChR.

[00168] Microtiter plates were coated with Torpedo AChR (1 µg/ml) in PBS and incubated overnight at 4°C. After blocking of the plates, mAb 198 preincubated in the presence of different concentrations of recombinant proteins, was added to the wells. Bound mAb 198 was detected by incubation with alkaline phosphatase-conjugated goat anti-rat IgG (1:10,000 dilution), followed by determination of alkaline phosphatase activity.

Induction and clinical evaluation of EAMG

[00169] Rats were immunized once in both hind foot pads by s.c. injection of Torpedo AChR (45 µg/rat) emulsified in CFA containing additional Mycobacterium tuberculosis (1mg/rat; Difco Labs, Detroit, MI). Clinical severity of EAMG was graded as follows: grade 0, rats with normal muscle strength; grade 1, mildly decreased activity, weak grip, with fatigability; grade 2, weakness, hunched posture at rest, decreased body weight, tremor; 3, severe generalized weakness, marked decrease in body

weight, moribund; 4, dead. Animals were evaluated weekly for 7-10 weeks following immunization with Torpedo AChR.

Induction of oral tolerance

[00170] Feeding with the recombinant fragments was initiated at the acute phase of EAMG, 7-10 days after immunization with Torpedo AChR and continued twice a week until the end of the experiment. The amount of recombinant fragments, and of thioredoxin (Trx) and ovalbumin (OVA, as control), was 600 µg/dose/rat in 1ml Tris buffer (50mM, pH 8.0).

Anti-AChR Ab assay

[00171] Antibodies to rat muscle AChR were measured by radioimmunoassay with crude rat muscle extract in which the AChR is specifically labeled by ^{125}I-α-BTX (Souroujon et al., 1983). Results are expressed as nmols antibody/L serum.

Lymphocyte proliferation assay

[00172] Draining lymph node cells (LNC) were cultured ($5x10^5$/well) in RPMI 1640 medium supplemented with HEPES, sodium pyruvate, glutamine, 2-ME, antibiotics, nonessential amino acids and 0.5% normal rat serum, either alone or in the presence of Torpedo AChR, Trx-Hα1-210, Hα1-205, or Con A. Proliferation was assessed by measuring (^{3}H)-thymidine (0.5µCi/well) incorporation during the last 18 h of a 4-day

culture period. Results are expressed as Δcpm after subtraction of background of unstimulated cultures from stimulated lymph node cells.

B-cell proliferation assay based on alkaline phosphatase activity

[00173] B-cell proliferation was assayed as described (Hashimoto et al., 1986 and Kasyapa et al., 1992). Draining LNC ($1x10^6$/ml) were cultured in the medium used for lymphocyte proliferation supplemented by 10% FCS. The cells were stimulated *in vitro* with Torpedo AChR (0.01µg/ml), Trx-Hα1-210 (50µg/ml), Trx (50µg/ml), Hα1-205 (50µg/ml), ConA (2µg/ml) or LPS (5µg/ml) in 24-well plates. After 4 days in culture, the cells were harvested, washed and diluted in PBS. For the alkaline phosphatase assay, 100 µl cell suspensions, containing different cell concentrations, were transferred to 96-well plates into which 100 µl/well of substrate solution (p-nitrophenyl phosphate, disodium; 1 mg/ml) was added. The plates were incubated for 2 h at 37°C in 5% CO_2. The optical density (O.D) at 405nm was measured and the data are expressed as O.D at 405nm per number of cells/well.

Determination of cytokines and costimulatory factors

[00174] PCR-ELISA was used to assess the levels of mRNA specific for cytokines (IL-2, IL-10, IL-12, IFN-γ and TGF-β) and costimulatory factors (CD40, CD40L, CD28, CTLA4, B7-1 and B7-2). RNA extraction, cDNA synthesis and RT-PCR in the presence of digoxigenin (DIG)-dNTP were performed as described (Zipris et al., 1996) with some modification suggested by the manufacturer of the PCR-ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany).

[00175] The sequences of primer pairs specific for rat IL-2, IL-10, IL-12, TGF-β, IFN-γ and β-actin were the same as previously reported (Im et al., 1999). The primer sequences specific for rat costimulatory factors and mouse CD40 are as follows; CD40 sense primer CGCTATGGGGCTGCTTGTTGACAG (SEQ ID NO:15); CD40 antisense primer GACGGTATCAGTGGTCTCAGTGGC (SEQ ID NO:16); CD40 internal primer CAGCCCAGTGGAACAGGGAGATTCGC (SEQ ID NO:17); CD40L sense primer 5'-GATCCTCAAATTGCAGCACA-3' (SEQ ID NO:18); CD40L antisense primer 5'-AGCCAAAAGATGAGAAGCCA-3' (SEQ ID NO:19); CD40L internal primer 5'-TGGGAGACAGCTGACGGTTAAAAG-3' (SEQ ID NO:20); CD28 sense primer 5'-CGGGAATGGGAATTTTACCT-3' (SEQ ID NO:21); CD28 antisense primer 5'-TCCAGAGCAGTGATGGTGAG-3' (SEQ ID NO:22); CD28 internal primer 5'-AACATGACACCGCGGAGACTCGGG-3' (SEQ ID NO:23); CTLA4 sense primer 5'-AGGACTTGGCCTTTTGGAGT-3' (SEQ ID

NO:24); CTLA4 antisense primer 5'-CAGTCCTTGGATGGTGAGGT (SEQ ID NO:25); CTLA4 internal primer 5'-TGATGAGGTCCGGGTGACGGTGCT-3' (SEQ ID NO:26); B7-1 sense primer 5'-GTGAGAGAAAAGGCATTGCTG-3' (SEQ ID NO:27); B7-1 antisense primer 5'-GGTTCTTGTTTGTTTCTCTGC-3' (SEQ ID NO:28); B7-1 internal primer 5'-GGTGCTCTCTGTCATCTCCGGGGT-3' (SEQ ID NO:29); B7-2 sense primer 5'-GAGGCAAGCTTACTTCAATAGCA-3' (SEQ ID NO:30); B7-2 antisense primer 5'-ATGCCAGTGTTTCTTGTTTCATT-3' (SEQ ID NO:31); B7-2 internal primer 5'-ACACCCACGGGATCAATTATCCTC-3' (SEQ ID NO:32).

[00176] The internal primers were all biotinylated by Biotin-Chem-Link (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. The amplified DIG-labeled PCR products were quantified using a PCR-ELISA kit. They were then denatured and hybridized to the suitable cytokine- or costimulatory factor-specific biotinylated internal primers for 3 h at 37°C with constant shaking. The DIG-labeled PCR product/biotinylated probe hybrids were immobilized on streptavidin-coated 96 well ELISA plates. After washing, the bound PCR products were detected with a peroxidase-conjugated anti-DIG antibody. PCR products were viewed with the peroxidase substrate ABTS, and signals were quantified by absorbance at 405 nm (Tsuruta et al., 1995).

Statistical analysis

[00177] Student's two-tailed T test was used to determine the significance of differences between group means.

RESULTS

Immunochemical characterization of AChR-derived recombinant fragments

[00178] The AChR-derived recombinant fragments of human AChR α-subunit were cloned and expressed either as fusion proteins with thioredoxin (Trx-Hα1-210) or glutathione-S-transferase (GST-Hα1-210), or without a fusion partner (Hα1-205). The extent of their conformational similarity to intact AChR was established by reactivity with α-BTX and mAb 198, an anti-AChR mAb specific for the main immunogenic region (MIR) in the α-subunit which is known to be a conformation-dependent epitope (Fig. 14B and 14C). As shown in Fig. 14B, Trx-H□1-210 binds α-BTX to a higher extent than the other two fragments. The weakest α-BTX binder was fragment Hα1-205. Denaturation of Hα1-205 by chemical modification completely abolished its ability to bind α-BTX, assessing the importance of conformation for this binding (data not shown). Similar results were obtained when the blot was overlaid with mAb 5.5 (Mochly-Rosen et al., 1981) which is directed to the acetylcholine binding site (data not shown). The anti-MIR mAb 198 (Tzartos et al.,

1981) bound well to Trx-Hα1-210 and, to a lower extent, to the other two fragments (GST-Hα1-210 and Hα1-205) (Fig. 14C).

[00179] The binding of the recombinant fragments to mAb 198 in Western blots was correlated with their ability to inhibit the binding of mAb 198 to Torpedo AChR in solution. As shown in Fig. 15, Trx-Hα1-210 inhibited this binding with an IC_{50} value of $3.0x10^{-7}$ M. The IC_{50} values for fragments GST-Hα1-210 and Hα1-205 were $1.3x10^{-6}$ M and $3.3x10^{-6}$ M, respectively. In all further experiments, the present inventors focused on two out of the three fragments, which represent the extremes with regard to conformational similarity to intact AChR. Namely, the *'more native'* fragment, Trx-Hα1-210 and the *'less native'* fragment, Hα1-205.

Effect of oral treatment with recombinant fragments on ongoing EAMG

[00180] The role of tolerogen conformation in modulation of EAMG was tested by oral administration of the fragments during the acute phase of disease in rats. The fragments tested were Trx-Hα1-210, Hα1-205 and their respective chemically modified forms, denTrx-Hα1-210 and denHα1-205. OVA and Trx alone were used as controls. Oral administration of the fragments was initiated at the acute phase, 8 days after the induction of EAMG, and was continued twice a week for 9 weeks. Treatment with Trx-Hα1-210 led to aggravation of disease

symptoms even as compared with control OVA-treated rats (Fig. 16). In the first five weeks after induction of disease, all rats treated with Trx-Hα1-210, got sick and 6 out of 10 died of EAMG. At that time, 3 out of 10 OVA-treated rats had died of EAMG whereas Hα1-205-treated rats showed only mild symptoms of EAMG (Fig. 16). Interestingly, oral treatment with the chemically modified, denatured form of Trx-Hα1-210, denTrx-Hα1-210, suppressed EAMG in a similar manner to Hα1-205 (data not shown). Treatment with Trx alone had no effect on EAMG (data not shown), assessing that the fusion partner (Trx) was not responsible for the aggravation of EAMG observed in the Trx-Hα1-210-treated rats. By ten weeks after disease induction, 7/10 rats in the Trx-Hα1-210-treated and 6/10 in the OVA-treated group were dead (The mean clinical scores were 3.4 for the Trx-Hα1-210-treatd group and 3.2 for the OVA-treated groups). On the other hand, in the Hα1-205-treated group, 3/10 rats were completely healthy and none of the rats died (mean clinical score: 1.3; Table 4).

Table 4 Effect of oral treatment with AChR fragments on ongoing EAMG ; Acute phase treatment

Trearment	Clinical score[a] 0	1	2	3	4 (No/total)	Mean clinical score[a]	Δ weight 3-8weeks (gr)	AChR content[b] (fmoles/mg prot.)	(%)	Anti-rat AChR[c] (nM)	T-cell proliferation (cpm)[d]
OVA	0/10	1/10	2/10	1/10	6/10	3.2	-11±11	20.5±3.5	45	70.5±6.5	4241±580
Trx-Hα1-210	0/10	1/10	1/10	1/10	7/10	3.4	-27±10	14.1±3.0	31	93.5±5.5	5205±640
Hα1-205	3/10	3/10	2/10	2/10	0/10	1.3	13±10	40.1±3.8	90	31.0±3.5	964±250

[a]Evaluated 10 weeks after the induction of EAMG

[b]Muscle AChR content in normal, age matched rats was 45.5±3.0 fmoles/mg protein and was referred to as the 100% value for this experiment.

[c] Evaluated 6 weeks after the induction of EAMG and derived from nmoles of α-BTX-binding sites precipitated by one liter of serum.

[d]In response to Torpedo AChR (0.25μg/ml)

[00181] The evaluation of clinical symptoms of rats treated with different proteins was corroborated by the analyses of muscle AChR content and body weight changes of the rats (Table 4). Rats in the Trx-Hα1-210 and control OVA-treated groups lost 69% and 55% of their muscle AChR content, respectively. In contrast, rats treated by Hα1-205 lost only 10% of their muscle AChR (Table 4). It should be noted that continuous long term oral administration to naive rats (for at least three months) of all tested recombinant fragments has never led to the development of clinical signs of EAMG. However, feeding with the 'more native' fragment Trx-Hα1-210 led to elicitation of antibodies to the fragment itself, whereas feeding with Hα1-205 or OVA did not elicit an antibody response to the fed antigen (data not shown).

[00182] Oral administration of the fragments was accompanied by different effects on AChR-specific humoral and cellular immune responses. Rats treated orally with Trx-Hα1-210 resulted in an increase in their anti-self AChR antibody levels (93.5±5.5 nM) when compared with the OVA-treated group (70.5±6.5 nM). On the other hand, treatment with Hα1-205 resulted in a decrease in the anti-self AChR antibody level (31.0±3.5 nM). In addition, Trx-Hα1-210-treated rats exhibited also a high AChR-specific proliferative T-cell response,

similar to the response in the OVA-treated rats, whereas Hα1-205-treated rats had a suppressed T-cell response (Table 4).

Effect of tolerogen conformation on the expression of cytokines and costimulatory factors

[00183] In order to analyze the possible mechanisms underlying the effects that the different fragments exert on EAMG, the levels of cytokines and costimulatory factors were studied in the treated rats. Draining lymph node cells of rats fed with Hα1-205, Trx-Hα1-210 or OVA were removed 5-8 weeks after EAMG induction and cultured for 48 h in the presence of Torpedo AChR. Total RNA was then prepared from the cells and subjected to PCR-ELISA with cytokine-specific or costimulatory factor-specific primers.

[00184] As shown in Fig. 17A, oral treatment with Trx-Hα1-210 resulted in down regulation of IFN-γ, IL-10 and TGF-β and up-regulation in the level of IL-2 (and a slight increase in IL-12) compared with OVA-treated rats. On the other hand, oral treatment with Hα1-205 resulted in suppression of Th1 type (IL-2, IL-12 and IFN-γ) cytokine mRNA levels and in up-regulation of Th2 type (IL-10) or Th3 type (TGF-β) cytokine mRNA levels as already reported by us (Im et al., 1999).

[00185] The observed stimulation of AChR-specific T-cell proliferation (Table 4) and up-regulation of Th-1 type cytokine levels (Fig. 17A) suggest alterations in the level of

costimulation in Trx-Hα1-210-treated rats. The expression levels of costimulatory factors were tested in the AChR-stimulated LNC which were used for analysis of cytokine levels. As shown in Fig. 17B, oral treatment with Trx-Hα1-210 resulted in up regulation of CD28, CD40 and CD40L compared with OVA-treated rats ($p < 0.005$). Other costimulatory factors such as CTLA4 and B7-1/B7-2 were similarly expressed in Trx-Hα1-210 and OVA-treated rats. On the other hand, oral treatment with the 'less native' fragment Hα1-205, which has been an effective tolerogen, resulted in reduced expression levels ($p < 0.005$; as compared to controls) of the costimulatory factors tested, such as CD40L, CD40, CD28, CTLA4 and B7-1/B7-2. This suggests that up-regulated expression of costimulatory factors induced by feeding with Trx-Hα1-210 leads to the increased AChR-specific T-cell proliferation. This activation of autoregulatory T-cells results in up-regulated Th1-type cytokines and down-regulation of Th2 or Th3 cytokines. On the other hand, the protective effect of oral treatment with Hα1-205 is accompanied by down-regulation of costimulatory factor expression, which in turn induces a suppressed AChR-T-cell response.

Effect of tolerogen conformation on T and B cell proliferation

[00186] In order to examine whether the observed upregulation of Th1-type cytokines and of costimulatory factors induced by Trx-Hα1-210 feeding, may be also associated with an increased AChR-specific B-cell proliferation, were compared the *in vitro* response of cells from myasthenic rats to the various fragments. Draining LNC were removed from myasthenic rats (mean clinical score: 2-3) at the chronic stage of disease, 6-8 weeks after EAMG induction. Cells were cultured for 4 days in the presence of Torpedo AChR, Trx-Hα1-210, Hα1-205, Trx, Con A or LPS and the level of B-cell proliferation was determined by alkaline phosphatase activity (which is known to be specific for activated B-cells; Hashimoto et al., 1986 and Kasyapa et al., 1992). Trx-Hα1-210 induced the highest B-cell proliferative response (Fig. 18A), whereas Trx alone had only a minor effect on B-cell proliferation. LPS induced a strong response and ConA did not induce any B-cell proliferative response (data not shown), as expected for activated B-cells. Interestingly, Torpedo AChR induced a lower B-cell proliferation than Trx-Hα1-210, which may be due to its processing *in vitro*.

[00187] T-cell proliferation was also assessed in the same LNC. As shown in Fig. 18B, T-cell proliferation in the presence of Trx-Hα1-210 was higher than in the presence of the

other fragments. Trx alone induced only a minor T-cell proliferation (data not shown). The different T-cell responses induced by the two fragments (Trx-Hα1-210 and Hα1-205), may reflect differences in their antigen processing and presentation in the LNC of myasthenic rats.

DISCUSSION

[00188] This example focuses on the role of conformation of orally administered AChR fragments in the induction of systemic suppression of EAMG. Insight was gained on the immunological pathways that follow the oral administration of conformationally different AChR fragments and this also suggest clues to predict what is required from a fed protein to serve as a successful tolerogen.

[00189] Rats were fed at the acute phase of EAMG with recombinant fragments, all corresponding to the extracellular domain of the human AChR α-subunit, but differing in their spatial conformation. One of the fragments, Hα1-205 was previously shown by the laboratory of the present inventors to suppress EAMG in rats when administered orally either at the acute or at the chronic phase of disease (Im et al., 1999). The other recombinant fragment, Trx-Hα1-210 corresponds to the same region in the human AChR α-subunit but in contrast to Hα1-205, its 3-D structure is more similar to that of the

corresponding region in native intact AChR. This was assessed by its reactivity with α-BTX, mAb 5.5 and mAb 198, all of which are known to recognize conformation-dependent epitopes of AChR. Another recombinant fragment consisting of the same sequence joined to GST (GST-Hα1-210) had intermediate characteristics. The present inventors have demonstrated that in contrast to Hα1-205 that suppresses EAMG, the 'more native' fragment, Trx-Hα1-210, fails to do so.

[00190] The next goal was to analyze the immunological events that follow the oral administration of these conformationally different fragments, and that result in one case in suppression and in the other case in exacerbation of an existing disease. The present inventors demonstrate that whereas the 'less native' fragment, Hα1-205 leads to a decreased humoral and cellular AChR-specific response accompanied by a decrease in the production of pro-inflammatory cytokines and costimulatory factors, the oral administration of the 'more native', Trx-Hα1-210 fragment leads to opposite changes. Namely, feeding with Trx-Hα1-210 leads to an elevated AChR-specific humoral and cellular reactivity and to an upregulation of the pro-inflammatory cytokine IL-2 and costimulatory factors accompanied by down-regulation of anti-inflammatory cytokines. Although Trx has been shown to act as a potent chemoattractant and inducer of

cytokines (Schenk et al., 1996 and Bertini et al., 1999), the latter effects cannot be attributed to Trx since denatured Trx-Hα1-210 and Trx alone did not act like Trx-Hα1-210.

[00191] Previous reports have demonstrated the involvement of the pro-inflammatory cytokines IL-12 and IFN-γ in the induction of EAMG (Balasa et al., 1997; Zhang et al., 1998 and Moiola et al., 1998) and the protective effects of anti-inflammatory cytokines such as IL-10 and TGF-β in autoimmune diseases including EAMG (Xiao et al., 1997). Therefore our observations on the different changes in the cytokine profile following the administration of Hα1-205 and Trx-Hα1-210, may explain the different effects of these two fragments on the course of EAMG.

[00192] The opposite consequences of oral administration of fragments differing in their conformation may stem from the repertoire of T and B cell epitopes they are bearing. The 'more native' fragment, Trx-Hα1-210 may be recognized by autoreactive B cells already existing in the myasthenic rats, that could serve as antigen-presenting cells required for T-cell activation, as has been implied in other autoimmune diseases (Falcone et al., 1998). Such a fragment is more likely to have deleterious effects upon oral ingestion. The 'less native' fragment, Hα1-205, probably bears significantly less, or no pathogenic B-cell epitopes at all,

and would therefore not stimulate B-cell proliferation that would in turn lead to AChR-specific T-cell activation. Our B-cell proliferation assay indeed demonstrates that Trx-Hα1-210 can stimulate B cells from sensitized rats whereas Hα1-205, denatured Trx-Hα1-210 and Trx alone, do not. Moreover, oral administration of Trx-Hα1-210 leads to increased levels of CD40L, which is expressed on activated T cells and is known to be an important costimulatory factor in B-cell activation. This factor has also been shown to be essential for AChR-specific immune responses since CD40L-deficient mice (CD40L -/-) are resistant to EAMG induction (Shi et al., 1998). The B-cell activation following the administration of a native AChR fragment could lead to the elevated AChR specific T-cell proliferation (Table 4) and to the observed shift in the cytokine profile from the desired Th2/Th3 response to the myasthenogenic Th1-regulated AChR-specific response. Conversely, when a less native AChR fragment, such as Hα1-205, is orally administered, the level of costimulation is too low to stimulate T-cell activation thus leading to a shift in the cytokine profile in favor of the anti-inflammatory Th2/Th3 cytokines.

[00193] In the present study, the present inventors have attempted to induce tolerance when EAMG already exists. In our experimental model, native conformation of the

tolerogen employed was not beneficial for the induction of oral tolerance. This might be due to some residual pathogenicity which may result in stimulation of already activated B cells, especially in the case of a highly immunogenic autoantigen as AChR. It is therefore important to delineate the requirements for an effective tolerogen. In the case of EAMG, the present inventors believe that myasthenogenicity of the tested fragments upon active immunization provides one such clue. Injections of large amounts of Trx-Hα1-210 (500 µg/dose in CFA) was observed to result in clinical signs of EAMG, while injection of the same dose of Hα1-205 was observed to result only in a transient disease characterized by very mild symptoms (mean clinical score: 1). Nevertheless, it should be stressed that even long term oral administration of any of the tested fragments never led to clinical signs of EAMG. Another clue is based on the ability to elicit a humoral response to the fed fragment. Oral feeding with the 'more native' fragment Trx-Hα1-210 led to production of anti-fragment antibodies, whereas feeding with denTrx-Hα1-210 or Hα1-205 did not elicit any humoral response.

[00194] The molecular features required for immunopathogenicity and tolerogenicity may be distinct from each other, and there is an advantage to be able to control them as desired. This distinction may be particularly

important for attempts to induce tolerance in an already existing disease. So far, most of the oral tolerance studies in experimental autoimmune diseases describe prevention experiments in which the tolerogen was introduced prior to disease induction, when antigen-specific activated B or T cells still do not exist. It may therefore be somewhat misleading to design clinical trials on the basis of such prevention studies. Moreover, this may be one of the reasons why clinical trials on ongoing human autoimmune diseases have not been very successful.

[00195] In conclusion, this study suggests that the spatial conformation of an orally administered tolerogen should be given careful attention when considering oral treatment for the induction of systemic tolerance in established antibody-mediated autoimmune diseases such as myasthenia gravis.

[00196] Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

[00197] While this invention has been described in connection with specific embodiments thereof, it will be

understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

[00198] All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by references.

[00199] Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

[00200] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the

skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

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